



INHIBITION OF SWARMING AND BIOFILM FORMATION BY *PROTEUS MIRABILIS* IN THE PRESENCE OF COMPOUNDS ISOLATED FROM *EICHHORNIA CRASSIPES*

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ABSTRACT

Water hyacinth (*Eichhornia crassipes*) is an invasive aquatic weed known to out-compete native plants. In this study, organic extracts of the floating plant parts were evaluated for their antibacterial and anti quorum sensing (QS) activity. However, the organic and aqueous extracts were found to possess no antibacterial activity against gram positive (*Bacillus subtilis*, *Enterococcus faecalis*) and gram negative (*Escherichia coli*, *Proteus mirabilis*) bacteria. The acetone extract of *E. crassipes* was found to exhibit anti QS activity, using *P. mirabilis* as the biosensor organism. Purification of the acetone extract by preparative thin layer chromatography (TLC) yielded two bioactive fractions, C and E. On comparison of the R_f and λ_{max} values of the potent fractions with published data of *E. crassipes* compounds purified under identical conditions, fractions C and E have been preliminarily identified as undecanoic acid and phenyl propanoid respectively. Two of the QS regulated virulence factors, viz, swarming and biofilm formation by *P. mirabilis* were significantly reduced in the presence of these fractions. This suggests that *E. crassipes* extracts contain compound(s) that could be used as anti-pathogenic agents.

KEYWORDS: *Eichhornia crassipes*, anti QS property, swarming, biofilm, TLC.

1. INTRODUCTION

Quorum sensing (QS), a population density dependent communication mechanism present in many bacteria, is mediated through small signal molecules called autoinducers that regulate the target gene expression responsible for the phenotypes essential for pathogenicity/symbiosis.^[1] There are many types of signaling molecules regulating diverse phenotypes, including the N-acylhomoserine lactones (AHLs), fatty acid derivatives, 4-quinolones, furanones (such as autoinducer-2 (AI-2) and oligopeptides.^[2] Detailed studies have revealed that QS plays a pivotal role in controlling the production of virulence factors such as exopolysaccharide and exoenzyme synthesis, biofilm formation, swarming motility, pigment and antibiotic production in several pathogenic bacteria.^[3] Plant metabolites have been traditionally used to treat human diseases including microbial infections. However, the mode of action of many phytochemicals against the target organism is not clearly understood, as the focus so far has been largely on the isolation of microbiocidal or microbiostatic compounds.^[4] Recent research has revealed that a few natural products including plant extracts have properties for modulating bacterial QS systems, thereby reducing the virulence of pathogens without inhibiting their growth.^[5] Such QS inhibitory or quorum quenching compounds, unlike conventional

antibiotics, are less likely to impose a selective pressure for the emergence of drug resistant bacteria.^[6] *Eichhornia crassipes*, commonly known as water hyacinth, is a water weed, known to be responsible for causing choking and eutrophication of water bodies worldwide. The floating plant parts contain several bioactive metabolites like alkaloids, phenolics, flavonoids and glutathione^[7] and have been used in traditional and folk medicine for the treatment of infections and its wound healing properties.

It is speculated that unlike humans and mammals that possess immune systems to defend against invaders, plants, which lack such sophisticated mechanisms, may have evolved to produce anti-microbial/ anti-QS compounds to defeat pathogens. Aquatic plants complete their entire life cycle in water, thus providing an ideal surface for biofilm formation. However, the occurrence of biofilm or infection by plant pathogens is rarely observed in water weeds as they may be endowed with effective defense mechanisms such as QS inhibition through metabolite production to avert biofouling. The first QS inhibitory compounds, the halogenated furanones were isolated from a marine red macro alga, *Delisea pulchra* and these interfere with the N-acyl homoserine lactone (AHL) regulatory system in several Gram-negative bacteria.^[8] These furanones actually

enable the alga to control bacterial colonization and fouling on its own surface in the natural marine environment. A recent study has revealed the presence of QS inhibitory compounds from other aquatic plants such as the water lily, *Nymphaea tetragona*^[9] and another marine alga, *Asparagopsis taxiformis*.^[10] However, there is a need for screening more aquatic plants for identification of novel QS inhibitors. Hence *E. crassipes* (water hyacinth), a prolific pest weed which is extensively used in folk medicine to control infections was selected for the present study as it may synthesize potent bioactive compounds.

Proteus mirabilis was selected as a bioindicator as it is one of the leading causative agents of catheter associated urinary tract infections which frequently develop into cystitis and pyelonephritis and can be further complicated by catheter encrustation and formation of urinary stones.^[11] On a suitable agar surface, *P. mirabilis* cells undergo repeated cycles of swarming migration and consolidation, giving rise to the typical 'bull's eye' swarm pattern characterized by circular symmetry and regularly spaced concentric terraces or zones.^[12] It has been shown that swarming motility may be the mechanism by which these pathogens gain entry into the host. *P. mirabilis* also produces several virulence factors responsible for its disease causing ability, which are known to be QS controlled.^[13]

In the present work, we have evaluated the antibacterial activity of *E. crassipes* extracts and the phytochemical constituents have also been analyzed. The major focus of this study, however, is on the inhibition of swarming motility and virulence factor production by *P. mirabilis* in the presence of *E. crassipes* solvent extracts as well as the anti-QS compounds purified from this plant using preparative thin layer chromatography.

2. MATERIALS AND METHODS

2.1 Microorganisms, Media and growth conditions

2.1.1 Microorganisms

Bacillus subtilis, *Enterococcus faecalis*, *Escherichia coli* and *Proteus mirabilis* were obtained from Institute of Microbial Technology (IMTech), Chandigarh, and routinely maintained on nutrient agar slants at 4°C. An overnight liquid culture of bacteria in Luria Bertani (LB) broth was used in all the experiments.

2.1.2 Media

Swarm agar: LB broth solidified with 1.5% agar was used for the swarm assays.^[14] The swarm agar plates (dried overnight at 37°C) were centrally inoculated with 5 µl of *P. mirabilis* cultures and incubated at 37°C for 24 hrs.

2.2 Plant material and extract preparation

The floating plant parts of *E. crassipes* were washed, air dried in the shade and ground to a fine powder. 5 g of the powder was subjected to successive extraction in a Soxhlet apparatus using solvents (150 ml each) with

increasing polarity, viz. petroleum ether, chloroform, acetone, ethyl acetate, and methanol. All the extracts were concentrated 10-fold and used for the assays. Controls were set up with each assay using an equal volume of the respective solvent.

2.3 Phytochemical Analysis

Qualitative analytical methods were used to identify the phytoconstituents present in the whole plant extracts. Tests were carried out for sterols, alkaloids, tannins, flavonoids and saponins.^[15]

2.4 Antimicrobial activity

The antibacterial activity of *E. crassipes* extracts was evaluated against gram positive (*B. subtilis*, *E. faecalis*) and gram negative (*P. mirabilis*, *E. coli*) bacteria by agar well diffusion^[16] method using Mueller-Hinton agar.

2.5 Anti-QS properties of *E. crassipes* extracts

P. mirabilis, an opportunistic pathogen often implicated in UTI was selected to study the effect of *E. crassipes* extracts on the production of some of its QS controlled virulence factors, viz. swarming and biofilm formation.

2.5.1 Swarm inhibition assay

To study the effects of *E. crassipes* extracts on the swarming motility of *P. mirabilis*, 5 µl of an overnight broth culture of the microorganism was centrally inoculated on swarm agar plates containing each of the extracts (10 µl/ml). A solvent control plate was also set up for each study; containing 10 µl/ml of the respective solvent. All the plates were incubated at 37°C for 24 hours. Thereafter, the diameter of the swarm zone in the experimental plates was measured and compared to the control.

2.5.2 Biofilm formation assay

The assay was performed using the micro titer plate method^[17] in the presence and absence of *E. crassipes* extracts (10 µl/ml). Briefly, overnight cultures of *P. mirabilis* grown statically in the presence and absence of plant extracts or purified TLC fractions in a 96-well plate were evaluated for the extent of biofilm formation by staining with 1% crystal violet, extracting the stain from the biofilm with solvent and measuring the absorbance at 450 nm. Wells containing an equal volume of the respective solvent served as control.

2.6 Isolation and identification of anti-QS compounds from the *E. crassipes* acetone extract

2.6.1 Preparative Thin layer chromatography (TLC)

Preparative TLC with pre-coated silica gel 60 F254 plates was used for the partial purification of anti-QS compounds from the *E. crassipes* acetone extract. The solvent system which provided the best resolution comprised of petroleum ether: ethyl acetate: Methanol (7:2:1).^[18] The developed plates were air-dried and the separated bands were detected based on their colors. The TLC was repeated several times and the mean R_f of the bands calculated. Five major fractions, A, B, C, D, and E

were identified and scraped out. The scraped silica was extracted with small volumes of the solvent system until stripped completely of color. The pooled extracts were evaporated to dryness, the residual purified fractions dissolved in an appropriate solvent and used for UV-Visible Spectroscopy and evaluation of anti QS properties.

2.6.2 UV-Visible Spectroscopy

The UV-Visible absorption spectra (190nm-1100nm) of the TLC purified fractions dissolved in chloroform were determined using a double beam bio-spectrophotometer, (BL-198, Elico Ltd.) with a chloroform blank for reference.

The R_f values of the purified fractions and the data obtained from UV-Visible spectroscopy was compared with the reference data in published literature.^[19]

2.6.3 Anti QS activity of partially purified fractions

The swarm inhibition assay was performed using the *E. crassipes* purified fractions A-E in order to identify the anti-QS compounds. LB agar plates were prepared containing each of the purified fractions at a concentration of 50µg/ml and dried overnight. 5µl of *P. mirabilis* culture was centrally spot inoculated and incubated at 37°C for 24hrs. Thereafter the diameters of the swarm zones in the presence and absence of the compounds were measured and compared. Further, these plates were used to estimate the concentration of N-Acyl-Homoserine lactone.

2.6.4 N-Acyl-Homoserine lactone assay

The QS signal compound used by *P. mirabilis*, N-acyl homoserine lactone was estimated in the presence and

absence of the *E. crassipes* purified fractions by the method used by Lépine, François, and Déziel.^[20] Briefly, swarm agar plates in the presence and absence of the purified fractions was cut into sections, submerged into 40ml of 90% ethanol and extracted in a shaker incubator for 24hrs. Supernatant was concentrated to 1ml and appropriately diluted samples were used for colorimetric estimation of amino acids.

2.7. Statistical analysis

All experiments were performed in triplicate. Results were expressed as mean $\pm$ standard error. Statistical significance was analyzed using Students t test. A p value less than 0.05 was considered statistically significant.

3. RESULTS

3.1 Phytochemical Analysis

Phytochemical analysis of the *E. crassipes* extracts was carried out to determine the class of the potential bioactive compounds. The extracts showed the presence of sterols, alkaloids, tannins, flavonoids, anthocyanins, anthroquinones and glycosides (Table 1). Sterols and glycosides were found to be present in almost all the extracts except in methanol extract while tanins were present only in petroleum ether extract. Anthocyanins were present only in acetone extract. Flavonoids, terpenoids and alkaloids were present in the methanol extract. The presence of wide range of phytochemical constituent indicates that the plant could be used in a multitude of ways.

Table 1: Phytochemical analysis of *E. crassipes* extracts

TESTS	CRUDE EXTRACT				
	Pet. Ether	Chloroform	Acetone	Ethyl Acetate	Methanol
1. Flavonoids	-	-	-	-	+
2. Saponins	-	-	-	-	-
3. Quinones	-	-	-	-	-
4. Terpenoids	-	-	-	-	+
5. Sterols	+	+	+	+	-
6. Anthrocyanins	-	-	+	-	-
7. Alkaloids	-	-	-	-	+
8. Tanins	+	-	-	-	-
9. Phenols	-	-	-	-	-
10. Glycoside	+	+	+	+	-
11. Anthraquinone	-	-	-	-	+
12. Phlobotanins	-	-	-	-	-

3.2 Antimicrobial activity

The antibacterial properties of *E. crassipes* were evaluated against gram positive as well as gram negative bacteria such as *B.subtilis*, *E. coli*, *P. mirabilis* and *Ent.faecalis* at whole plant extract concentrations of 10–50 µg/ml. None of the *E. crassipes* extracts were found

to possess any antibacterial activity at these concentrations.

3.3 Anti-QS properties

Swarming and biofilm formation in *P. mirabilis* are controlled by QS. These activities were evaluated in the

presence of *E. crassipes* extracts as well as purified fractions.

3.3.1 Effect of *E. crassipes* extracts on swarming motility

The plant extracts in various solvents were found to differ in their potency with respect to swarming inhibition, exhibiting 10%-60% reduction in the diameter of the swarm zone of *P. mirabilis*. Apart from this obvious inhibition of swarming motility, it was observed that there was an alteration in the swarm pattern of *P. mirabilis* in the presence of the extracts- the width of the individual swarm rings increased along with a concomitant decrease in the number of rings. The

acetone extract proved to be the most effective in inhibiting the swarming motility of *P. mirabilis* and was therefore selected for further study. Fig.1 depicts the considerable (30%) reduction in the diameter of the swarm zone of *P. mirabilis* in the presence of acetone extract. Swarm agar plates containing a gradient of the acetone extract (10 μ l-70 μ l/ml) were set up to observe the changes in swarm pattern and reduction in swarm zone. A significant ($p < 0.04$) dose dependent decrease in the overall diameter of swarm zone was observed in the presence of the acetone extract (fig. 2). The other extracts were found to be less effective in inhibiting swarming, showing 10-20% reduction in swarm zone diameter.



Figure 1: Effect of *E. crassipes* acetone extract on swarming motility of *P. mirabilis*.

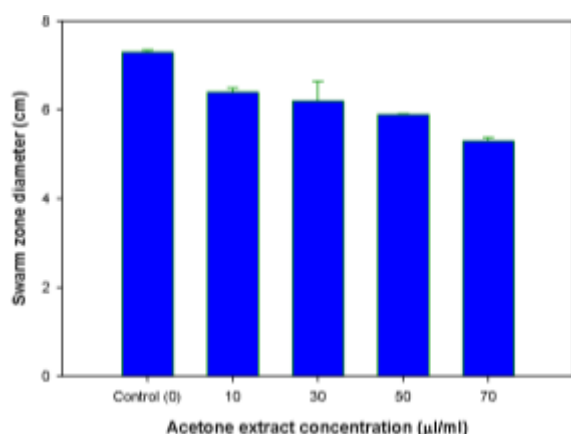


Figure 2: Dose dependent decrease ($P < 0.04$) in swarm zone of *P. mirabilis* in presence of acetone extract

3.3.2 Inhibition of biofilm formation

The absorbance of the extracted crystal violet is proportional to the cell biomass in the biofilm. The average absorbance level of crystal violet were found to be 0.043 ± 0.0172 , 0.035 ± 0.0005 , 0.048 ± 0.005 , 0.045 ± 0.002 , 0.046 ± 0.002 in the presence of petroleum ether, chloroform, acetone, ethyl acetate, methanol extracts respectively and 0.07 ± 0.028 , 0.04 ± 0.0028 , 0.051 ± 0.002 , 0.054 ± 0.007 , 0.065 ± 0.008 in the respective solvent control wells. The absorbance levels in wells containing *E. crassipes* extracts are remarkably lower as compared to the solvent control wells containing the respective solvents (fig. 3). Thus the biofilm forming capability of *P. mirabilis* seems to be affected by the

bioactive components in the extracts. Biofilm formation was found to be significantly reduced in the presence of the acetone extract of *E. crassipes*.

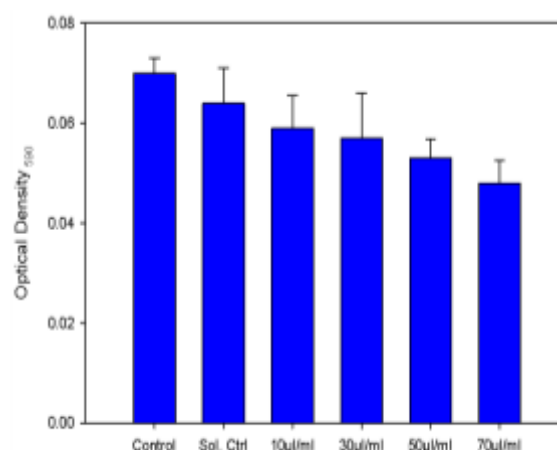


Figure 3: Dose dependent reduction ($P < 0.03$) in biofilm formation by *P. mirabilis* in presence of acetone extract.

3.4. Thin layer chromatography (TLC)

The acetone extract was subjected to preparative TLC using several solvent systems. The best resolution was obtained with a predominantly non-polar solvent system made up of Pet. Ether: Ethyl acetate: Methanol (7:2:1) and five distinct bands (A-E) were obtained. The colors and R_f values of the bands are given in Table 2. A polar compound which did not migrate remained at the spot.

This spot was scraped, extracted and run in a solvent system comprising of Pet. Ether: Ethyl acetate: Methanol (2:3:5). It resolved as a single spot in this predominantly polar system.

3.5 Ultraviolet-Visible Spectrophotometry

The fractions purified by preparative TLC were suspended in chloroform and subjected to UV-visible absorption spectroscopy using the solvent chloroform as blank. The $\lambda_{\max}$ values were noted and are depicted in Table 2.

Table 2: R_f values and UV-Vis $\lambda_{\max}$ of the TLC fractions of *E. crassipes* Acetone extract:

	Fractions	Color	R_f of the fractions	$\lambda_{\max}$	Predicted Compound ^a	Bioactivity(%)
	A	Dark green	0.92	293,375,413,666	Diterpenes	14%
	B	Light green	0.78	256,330,438,657	Campasterol	32%
	C	Faint green	0.68	263,396,410,666	Undecanoic acid	68%
	D	Faint yellow	0.47	296,430,661	Phthalic acid	18%
	E	Brown	0.36	295,415,662	Phenyl propanoid	72%

^aLalitha. *et.al* 2012

Solvent system: Pet. Ether: Ethyl acetate: Methanol (7:2:1).

3.6 Bioactivity of purified compounds

The anti-QS properties of the TLC fractions were estimated by measuring the swarm zone of *P. mirabilis* in the presence and absence of the fractions. Significant reduction in the diameter of swarm zone was observed in the presence of fractions C and E. (fig. 4). Maximum reduction in the diameter of swarm zone (66%) was demonstrated in the presence of fraction E.

3.7 Acyl-Homoserine lactone assay (AHL)

The QS signal molecule, AHL, produced by *P. mirabilis* was quantified in the presence and absence of fractions C and E in swarm plates. Along with a reduction in the diameter of swarm zone of *P. mirabilis*, a concomitant reduction in AHL concentration was observed in the presence of the bioactive fractions C and E (fig. 4). A 50% reduction in the AHL concentration was observed in the presence of fraction E whereas a 40% reduction in the AHL concentration was demonstrated in the presence of fraction C as compared to a control set-up lacking any additive.

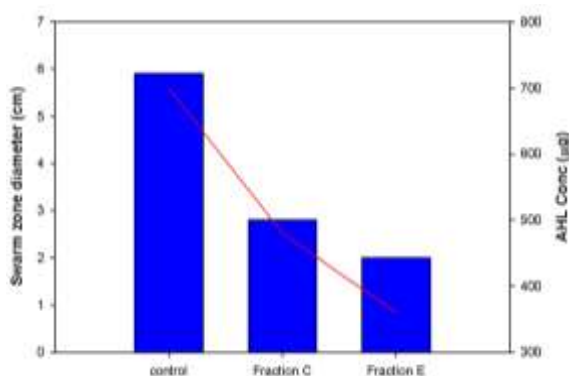


Fig 4: Effect of TLC purified fractions of *E. crassipes* on swarm zone diameter and AHL production by *P. mirabilis*

4. DISCUSSION

There have been several ethanobotanically directed searches for anti QS compounds^[21] capable of attenuating the virulence of pathogens without challenging basic cell function and growth, which would prevent the emergence of multiple drug resistant strains. However, the majority of such screening programmes mainly target dietary and medicinal plants. Pest weeds, both aquatic as well as terrestrial, grow prolifically and are rarely found to be infected by plant pathogens. Obviously, they possess efficient metabolites to ward off diseases. Hence *E. crassipes*, an invasive water weed which is used for its wound healing properties by tribals in Asia, was selected for this study as part of a screening programme in our laboratory with an objective to identify anti QS compounds. *P. mirabilis* was selected as the biosensor to study the anti QS properties of *E. crassipes*. UTI with *P. mirabilis* is known to ascend rapidly to the kidneys, which may lead to pyelonephritis, inflammation and renal failure.^[22] Several virulence factors such as swarming motility, urease and hemolysin production and biofilm formation ability may be responsible for its pathogenicity and the expression of all these is controlled by QS. Two of these virulence factors, swarming motility and biofilm formation ability, were selected in this work to assess the anti QS properties of *E. crassipes*.

4.1 Anti-microbial Activity

Plant extracts are potential sources of novel antimicrobial compounds especially against bacterial and fungal pathogens. It has been suggested that the antimicrobial activity is mainly due to the presence of essential oils, flavonoids and other natural polyphenolic compounds or free hydroxyl groups.^[23]

Although all the potent phytochemicals reported to possess antibacterial properties were found to be present

in the five solvent extracts of *E. crassipes*, none of these extracts exhibited conventional antibacterial activity against *B. subtilis*, *E. faecalis*, *E. coli*, *P. mirabilis* as judged by the cell viability (data not shown). Baral and Vaidya^[24] have also reported no antibacterial activity against these organisms. Obviously, the compounds present in the organic extracts are not detrimental to the basic metabolism and growth of these organisms and the plant possesses some other mechanism to ward off bacterial infections and biofilm formation on its surfaces.

4.2 Anti QS property

Since the *E. crassipes* extracts were not found to possess anti microbial properties, it seemed likely to possess anti QS properties which would prevent disease just as an anti-microbial compound without preempting the emergence of resistant bacterial strains. Swarming migration and biofilm formation was studied in the presence of these extracts at concentrations where no antimicrobial activity was observed.

4.2.1 Swarming motility

Swarming by *P. mirabilis* was found to be inhibited in the presence of all the *E. crassipes* extracts with maximum inhibition (60%) in the presence of the acetone extract. It was observed that not only was the diameter of the swarm zone significantly reduced ($p < 0.04$) in the presence of the acetone extract but the typical swarm ring pattern also changed. Echeverrigaray et al^[25] have reported a similar change in swarm pattern of *P. mirabilis* in the presence of pure terpenes. Swarming enables bacterial cells to migrate rapidly and collectively over large areas, greatly facilitating infection and colonization of surfaces. Allison and Hughes^[26] have demonstrated that the differentiated swarm cells of *P. mirabilis* are highly invasive and are responsible for the colonization of cells and ascending migration of bacteria against the flow of urine and mucous in UTI. Thus, a compound in the acetone extracts of *E. crassipes* which inhibits swarming would presumably be effective in controlling ascending UTI by *P. mirabilis*.

4.2.2 Biofilm formation

Since biofilm formation is regulated by QS, compounds possessing anti-QS activity would have a significant effect on the biofilm formation capability of *P. mirabilis*. Indeed, all the *E. crassipes* extracts affected a decrease in biofilm formation in vitro. As with swarming motility, maximum reduction in biofilm formation was observed in the presence of the acetone extract and hence this extract was selected for further study of biofilm inhibition using a concentration gradient. A dose dependent decrease in the extent of biofilm formation was observed with about 40% reduction in biofilm formation at a relatively low concentration of 70ul/ml. Disruption of the QS system with furanones has also been shown to inhibit biofilm growth.^[27] Biofilm formation and biofouling are global phenomena causing economic problems in medicine and industry. Quorum sensing inhibition (QSI) may represent a natural,

widespread, but non conventional antimicrobial strategy utilized by plants and other organisms with significant impact on biofilm formation.^[28]

4.3 Purification of bioactive compounds and evaluation of their anti QS properties

The phytochemistry of the solvent extracts is highly complex and may be composed of several compounds with varied modes of action which may act in concert. Dried and powdered *E. crassipes* was successively extracted in a series of solvents with increasing polarity which served as a preliminary step in the separation of polar and non-polar compounds. The acetone extract which exhibited maximum reduction in swarm zone as well as biofilm formation by *P. mirabilis* was selected for the isolation of bioactive compounds. The preparative TLC of the acetone extract yielded five distinct bands in a predominantly non-polar solvent system made up of Pet. Ether: Ethyl acetate: Methanol (7:2:1).

All the fractions, including the polar compound remaining at the spot, were subjected to swarming motility assay.

Fractions C and E were found to significantly ($p=0.02$) inhibit swarming motility of *P. mirabilis* – fraction E being the most potent, causing a 66% reduction in the diameter of the swarm zone. The R_f and λ_{max} values of the fractions were compared with the values documented in the literature for *E. crassipes* under the same operating conditions and a preliminary prediction of the class /compound was made.^[29] Accordingly, fraction E may be a phenyl propanoid and fraction C might be undecanoic acid, although further confirmation is necessary.

In order to further substantiate the anti QS properties of fractions C and E, AHL was quantified in the presence and absence of these fractions. A significant reduction in the AHL levels in the presence of fractions C and E definitively confirms their anti QS potential, although the exact mechanism of action cannot be predicted from this data. QS Inhibition occurs via a number of different mechanisms^[30] such as signal mimicry in the case of the furanones, signal degradation by proteins such as lactonase and acylase, signal binding, the inhibition of genetic regulation systems, or the interruption of downstream virulence and biofilm genes. Since the bioactive plant compounds have extremely complex structures, the exact mechanism of action cannot often be specified. An overall inhibition of the QS system was observed in the presence of the *E. crassipes* solvent extracts as well as the purified fractions which suggests that either the diverse chemicals in the plant may have varied effects on the QS by *P. mirabilis* or the effect of the extracts may be on a more global QS regulator, and not directly on the LuxI/LuxR system.

5. CONCLUSION

Aquatic plants live in environments where bacterial infections and biofouling can occur easily. Natural selection would therefore presumably impart some

protective mechanisms to these organisms, more so to invasive water weeds^[31] such as *E. crassipes* which are seldom infected by plant pathogens. Although *E. crassipes* extracts were not found to possess antimicrobial activity, the acetone extract was found to contain two potent anti QS compound (s) which have been preliminarily identified as a phenyl propanoid and undecanoic acid, although further confirmation is necessary. These compounds could be used as anti-pathogenic agents, quenching virulence without inhibiting basic cell functions, which would prevent the emergence of drug resistant strains. Several researchers have investigated the anti microbial, anti oxidant and wound healing properties^[32,33,34] of *E. crassipes*. However, to the best of our knowledge, this is the first study on the anti QS properties of this plant, effectively validating its traditional usage in folk medicine.

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